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Plague Pandemics Investigated by Ribotyping of *Yersinia pestis* Strains

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***Yersinia pestis* is the causative agent of plague, a disease which has caused the deaths of millions of people and which persists now in endemic foci. The rRNA gene restriction patterns (i.e., ribotypes) of 70 strains of *Y. pestis*, isolated on the five continents over a period of 72 years, were determined by hybridization with a 16S-23S rRNA probe from *Escherichia coli*. The combination of the *Eco*RI and *Eco*RV patterns resulted in the elucidation of 16 ribotypes. Two of them (B and O) characterized 65.7% of the strains studied, while the 14 other ribotypes were found in no more than three strains each. A relationship was established between biovars and ribotypes: strains of biovar Orientalis were of ribotypes A to G, those of biovar Antiqua were of ribotypes F to O, and those of biovar Medievalis were of ribotypes O and P. Great heterogeneity in rRNA restriction patterns was found among strains isolated in Africa; this heterogeneity was less pronounced among Asian isolates and was completely absent from the American strains. Pulsed-field gel electrophoresis was performed on the DNAs of some strains, but it appeared that different colonies from the same strain displayed different pulsed-field gel electrophoresis patterns and therefore that this technique was not suitable for comparison of *Y. pestis* isolates. In contrast, the ribotypes of individual colonies within a given strain were stable and were not modified after five passages in vivo. A clear correlation between the history of the three plague pandemics and the ribotypes of the strains could be established.**

Yersinia pestis, the plague bacillus, has been one of the most devastating infectious agents in world history. Cases of plague were described as far back as the pre-Christian era, but their numerical and historical importance remain unknown. Later, during the Christian era, three well-documented plague pandemics occurred (7, 19, 22).

The first pandemic, called Justinian's plague, probably started from Central Africa. It reached lower Egypt and then the Mediterranean countries during the 6th century. The pandemic lasted for 50 to 60 years, and it was estimated that 100 million people died (19). The second pandemic, known as the Black Death, originated in Central Asia around the Caspian Sea, reached the Crimean ports during the 14th century, and invaded all of Europe and the northern part of Africa. This epidemic became established during 4 centuries and killed one-quarter of the European population (19). Small endemic foci persisted in Central Asia and Central Africa. The third (and present) pandemic began in the Yunnan region of China and reached Hong Kong in 1894. The existence of steamships and railways greatly favored a rapid spread of the disease on the five continents and the colonization of new geographic areas (e.g., the United States, South America, South Africa, and Madagascar). The number of victims reported to date is not comparable to those during the two previous pandemics.

However, plague has not been, and will not be soon, eradicated, despite the major advances made since the beginning of this century in the knowledge of the disease, in public health, and in therapy. The reasons for the persistence of plague might be found in its epidemiology: plague is essentially a disease of wild rodents that is transmitted by fleas. The control of the animal population is extremely difficult, since the burrows are most often located in inaccessible areas. Furthermore, the complete destruction of the rodent population does not guarantee the extinction of the plague foci because *Y. pestis* can survive for several years in the litter of dead animals (13, 16) and then reinfect the new occupiers of the burrows (2). Nowadays, endemic foci of plague persist in Africa (Madagascar, Tanzania, Kenya, Zaire, Botswana, and Uganda), North and South America (Bolivia, Brazil, Peru, and the United States), and Asia (Vietnam, China, Mongolia, Kazakhstan, and Myanmar). Nineteen hundred sixty-six cases of human plague were reported to the World Health Organization in 1991. This number is the highest for the last 15 years (21).

Since its first isolation in Hong Kong in 1894 by Yersin, *Y. pestis* has been extensively studied. The main characteristics of the species are its low number of positive biochemical reactions and its great homogeneity: there is only one serotype, one phage type, and three biovars (7). This homogeneity is quite surprising, since the bacterium had to adapt to various types of mammalian hosts (e.g., rodents, camels, rabbits, and humans) and species of flea vectors, as well as to widely differing climates and geographical environments.

To further investigate the degree of homogeneity of the species *Y. pestis*, we decided to use and to compare two recent methods of bacterial typing: rRNA gene restriction patterns (ribotyping) and pulsed-field gel electrophoresis (PFGE). Ribotyping has been successfully applied to many bacterial species, including some *Yersinia* species (1, 4, 18), but has never been used for *Y. pestis*. Typing of *Yersinia enterocolitica* (17) and *Y. pestis* (15) by PFGE has been described, and it was of interest to determine which of the two methods was most suitable for the study of the plague bacillus.

In the present work, the rDNA polymorphisms of 70 strains of *Y. pestis* isolated during different periods and from various geographical areas were examined. We demonstrate that *Y. pestis* can be subdivided into 16 different ribotypes and that a link exists between the ribotypes and the biovars of the strains.

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We also show that there is great heterogeneity in the PFGE patterns (i.e., pulsotypes) within a given strain of *Y. pestis*, while its ribotype remains stable, both in vitro and after several passages in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In the present study, a total of 70 strains of *Y. pestis* from the collection of the French *Yersinia* Reference Laboratory (Institut Pasteur, Paris) were used (Table 1). These strains were isolated between 1908 and 1979 from 16 different geographical areas. The precise year of isolation of some strains is not known. On the basis of their abilities to ferment glycerol and to reduce nitrate, the *Y. pestis* strains were divided into three biovars: Antiqua (glycerol and nitrate positive), Medievalis (glycerol positive and nitrate negative), and Orientalis (glycerol negative and nitrate positive) (7).

To obtain genomic DNA, bacterial suspensions were prepared from stock cultures and were streaked on Trypticase soy agar plates. Plates were incubated at 25°C for 24 to 48 h, i.e., until confluent growth was visible.

DNA extraction, restriction, and transfer to nylon membranes. To extract total DNA from each *Y. pestis* strain, confluent lawns of bacteria were harvested from the plate and washed twice in saline. DNA extraction was performed as described previously (6), except that incubation in the lysis buffer lasted for 2 to 3 h instead of 24 h. The DNA concentration was measured by UV A_{260}.

DNAs of selected isolates were initially digested with five different restriction endonucleases: *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Bam*HI (Amersham International, Amersham, England). *Eco*RI and *Eco*RV appeared to be the most suitable enzymes and were used to cut DNAs from all strains studied. Five micrograms of each sample was digested for 4 h at 37°C before being loaded in a 0.8% agarose (Appligene, Illkirch, France) horizontal gel and subjected to overnight electrophoresis at 50 V in 1× TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Alkaline denaturation, neutralization, and transfer of DNA onto nylon filters (Hybond-N; Amersham) with a VacuGene apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) were performed as previously described (14).

The molecular weight standard used to determine the sizes of the DNA fragments was the *Eco*RI-digested genomic DNA of *Xenorhabdus* strain 278. The molecular size of each standard fragment is given in the legend to Fig. 1.

Preparation of radioactive probe and hybridization. The 16S plus 23S rRNA from *Escherichia coli* (Boehringer, Mannheim, Germany) was used as a probe and was radioactively labeled by 5′ end labeling with [γ-^{32}P]ATP (Amersham) and a 5′ DNA terminus labeling kit with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's recommendations. Hybridization of Southern blots to radioactive rRNA was performed as described previously (10) and was detected by autoradiography of the filters.

Hybridization with AAF-labeled probe. A nonradioactive acetylaminofluorene (AAF)-labeled 16S-23S rRNA probe (9) was also used in our study. Hybridization and immunoenzymatic detection of hybridizing fragments were performed according to the recommendations of the manufacturer (Eurogentec, Seraing, Belgium) with slight modifications; i.e., the wash solution was 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate instead of 2× SSC and the saturation buffer was 0.5% casein instead of 1% casein.

Determination of molecular sizes of the fragments. The migration of each hybridizing fragment in the gel was calculated with a Gel Reader (IBI, New Haven, Conn.). The sizes of these fragments were determined with the Mac Vector software (IBI). This software uses two methods in parallel to calculate molecular weight: the least-squares method based on the method of Schaffer and Sederoff (20) and the empirical cubic spline method.

PFGE. To perform PFGE, genomic DNAs of various strains of *Y. pestis* were prepared in agarose plugs as described previously (11) with minor modifications. The DNA samples were digested overnight at 37°C in 300 μl of the appropriate restriction buffer containing 10 U of either *Spe*I, *Xba*I, or *Not*I endonuclease. The DNA included in one-sixth of an agarose plug was subjected to zero integrated field electrophoresis with the AutoBase system (Q-Life Systems Inc., Kingston, Ontario, Canada). Electrophoreses were carried out in 0.8% agarose gels and in 1× TBE buffer for 65 h at room temperature, using ROM cards which allowed the optimal separation of fragments ranging from 50 to 100 kb or from 150 to 200 kb. Lambda oligomers (FMC Corp., Rockland, Maine) were used as size markers. Restriction fragments were visualized after the gels were stained with ethidium bromide.

Infection of mice and recovery of bacteria. Two 5-week-old OF1 female mice (Ifa-Credo) were infected subcutaneously with 10^3 *Y. pestis* Madagascar 6/69 organisms in 0.1 ml of saline. The spleens were removed 3 days later and crushed in 1.5 ml of saline, and serial dilutions were streaked on Trypticase soy agar plates containing 25 mM hemin. Hemin was added in the medium in order to obtain isolated colonies. After 48 h of incubation at 28°C, two isolated colonies were picked, their DNA was extracted for ribotyping, and 10^3 bacteria from each colony were injected in one mouse as described above. This procedure was performed five times.

RESULTS

Analysis of hybridization patterns. Five restriction enzymes were tested for the ability to yield the best DNA banding pattern. *Eco*RI and *Eco*RV were found to be the most appropriate restriction endonucleases for *Y. pestis*, and both of them were used to digest the chromosomal DNAs of the 70 strains listed in Table 1. Faint hybridizing bands which could not be systematically visualized were not taken into account. Hybridization with ^{32}P-labeled or AAF-labeled probes gave similar results, but, as noted previously (9), the DNA fragments recognized by the nonradioactive probe were sharper and therefore more distinctly differentiated.

When the DNA samples were digested with *Eco*RI, five to seven clearly visible restriction fragments were recognized by the rRNA probe (Fig. 1 and 2). Despite the existence of 11 different patterns (RI.1 to RI.11), the profiles obtained were relatively homogeneous, with most of them differing only by the presence or the absence of one restriction fragment (Fig. 2). Two *Eco*RI fragments of approximately 18.2 and 7.6 kb hybridized with the rRNA probe in all the *Y. pestis* strains studied. In addition, a hybridizing fragment of 4.7 kb was present in all strains tested except strain Senegal Fa (Fig. 2).

When the *Eco*RV enzyme was used, three to six DNA segments hybridized with the probe (Fig. 1 and 3). Similarly to the *Eco*RI patterns, the 11 *Eco*RV patterns displayed a certain degree of homogeneity. Two hybridizing *Eco*RV fragments of ca. 7.5 and 5.8 kb were common to the 70 *Y. pestis* strains, and

TABLE 1. Characteristics of the 70 strains of *Y. pestis* used for ribotyping

Geographic origin[a]	Strain	Biovar	Yr of isolation	*Eco*RI pattern	*Eco*RV pattern	Ribotype
Brazil	EXU 21	Orientalis	1967	1	2	B
	EXU 25	Orientalis	1967	1	2	B
	EXU 44	Orientalis	1967	1	2	B
	EXU 53	Orientalis	1967	1	2	B
	EXU 56	Orientalis	1967	1	2	B
	EXU 126	Orientalis	1967	1	2	B
	EXU 141	Orientalis	1967	1	2	B
	EXU 184	Orientalis	1967	1	2	B
United States	A1122	Orientalis	1947	1	2	B
	7793	Orientalis	1948	1	2	B
Hawaii	193	Orientalis	1950?	1	2	B
Java	TP	Orientalis	1920?	1	2	B
	TO	Orientalis	1929	1	2	B
Bombay	195	Orientalis	1908	1	2	B
Nhatrang	63-138	Orientalis	1963	1	4	G
	63-110	Orientalis	1963	1	4	G
Saigon	55-1239	Orientalis	1955	6	2	E
	55-800	Orientalis	1955	6	2	E
	55-801	Orientalis	1955	6	2	E
Turkey	10.1	Orientalis	UN[b]	1	2	B
	10.3	Orientalis	UN	1	2	B
	10.5	Medievalis	UN	4	5	N
Kurdistan	PKH III	Medievalis	1947	4	5	O
	PKH IV	Medievalis	1951	4	5	O
	PKR V	Medievalis	1947	9	11	P
	PKR VI	Medievalis	1947	4	5	O
	PKR VII	Medievalis	1947	4	5	O
	PKR VIII	Medievalis	1948	4	5	O
	PKR XVIII	Medievalis	1948	4	5	O
	PKR XXIV	Medievalis	1948	4	5	
	PKR XXV	Medievalis	1948	4	5	O
	PKR 288	Medievalis	1963	4	5	O
	PKR 290	Medievalis	1963	4	5	O
	PKR 292	Medievalis	1963	4	5	O
	PKR 298	Medievalis	1963	4	5	O
	PKR 299	Medievalis	1963	4	5	O
	PKR 300	Medievalis	1963	4	5	O
	PKR 301	Medievalis	1963	4	5	O
Harbin	UN	Antiqua	<1948	4	5	O
Kenya	129 M22	Medievalis	<1952	4	5	O
	MMI	Antiqua	1979	4	5	O
	DM	Antiqua	1979	4	5	O
	EV	Antiqua	UN	10	5	M
	RA	Antiqua	UN	10	5	M
	164	Antiqua	<1952	4	9	I
	129	Antiqua	<1952	4	9	I
	102	Antiqua	<1952	1	4	G
	147	Antiqua	<1952	3	7	H
	162	Antiqua	<1952	3	7	H
	169	Antiqua	<1952	3	8	K
	144	Antiqua	<1952	3	6	L
	Tanzania I	Antiqua	UN	2	5	F
	Ky	Antiqua	UN	2	5	F
	MM	Antiqua	UN	10	5	M
Belgian Congo	343	Antiqua	1941	11	5	N
	Li	Antiqua	<1953	5	10	J

Continued on following page

TABLE 1—*Continued*

Geographic origin[a]	Strain	Biovar	Yr of isolation	*Eco*RI pattern	*Eco*RV pattern	Ribotype
Madagascar	Ga	Orientalis	<1939	1	2	B
	62	Orientalis	1946	1	2	B
	112	Orientalis	1951	1	2	B
	6/69	Orientalis	1969	1	2	B
Morocco	243	Orientalis	1940	1	2	B
	48	Orientalis	1940	1	2	B
Senegal	Ro	Orientalis	1944	1	2	B
	Th	Orientalis	1944	1	2	B
	Fa	Orientalis	1944	8	3	D
Hambourg	12	Orientalis	UN	1	1	A
	19	Orientalis	UN	1	2	B
	5	Orientalis	UN	1	2	B
	13	Orientalis	UN	7	2	C
	11	Orientalis	UN	2	5	F

[a] The names of the countries or towns are those used at the time of the strain isolation and have been kept for strain designation.
[b] UN, unknown.

one fragment of 5.5 kb was found in 69 strains but not in strain Senegal Fa (Fig. 3).

Distribution of ribotypes among the 70 *Y. pestis* strains. Five *Eco*RI profiles were found in only one strain each, and the 65 remaining strains belonged to six different groups. RI.1 was the most frequent (30 of 70), followed by RI.4 (22 of 70). Therefore, 74.3% of the *Y. pestis* strains studied fell into two main patterns. Comparison of these two patterns indicated that they were identical except that one additional band was present in RI.4 (Fig. 2). Similarly, the majority of the strains (81.4%) had two main *Eco*RV profiles, RV.2 (30 of 70) and RV.5 (27 of 70), and the two patterns differed only by the presence of an additional *Eco*RV fragment in RV.5 (Fig. 3).

The results obtained with the two restriction enzymes were consistent: RI.1 was most often linked to RV.2, and RI.4 was most often linked to RV.5. However, strains Senegal Fa, Belgian Congo Li, and Kurdistan PKRV each had a unique and specific pattern with both *Eco*RI and *Eco*RV. These results suggest some parallelism in the conservation (or the loss) of the *Eco*RI and *Eco*RV sites on the rRNA operons of *Y. pestis*.

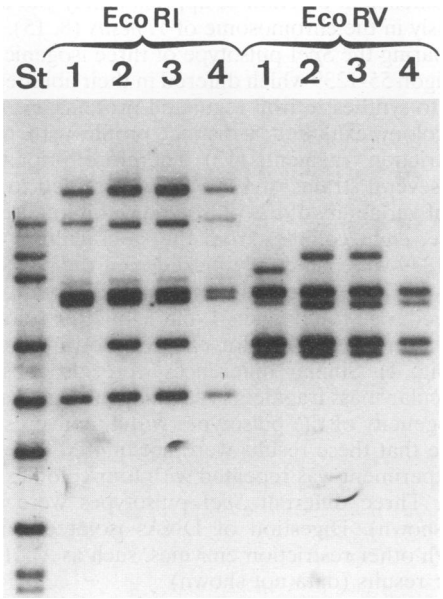

FIG. 1. Representative patterns of *Eco*RI- or *Eco*RV-digested genomic DNAs of four strains of *Y. pestis* hybridized with the *E. coli* 16S-23S rRNA ^{32}P-labeled probe. St, *Eco*RI-cleaved DNA of *Xenorhabdus* strain 278 used as a molecular mass standard; the sizes of the bands, from top to bottom, are 13.0, 11.6, 10.1, 8.5, 5.8, 4.6, 3.8, 2.9, 2.5, and 2.4 kb. Lanes: 1, Hambourg 12 (RI.1 plus RV.1); 2, Kurdistan PKR XXIV (RI.4 plus RV.5); 3, Kurdistan PKH III (RI.4 plus RV.5); 4, Madagascar 112 (RI.1 plus RV.2).

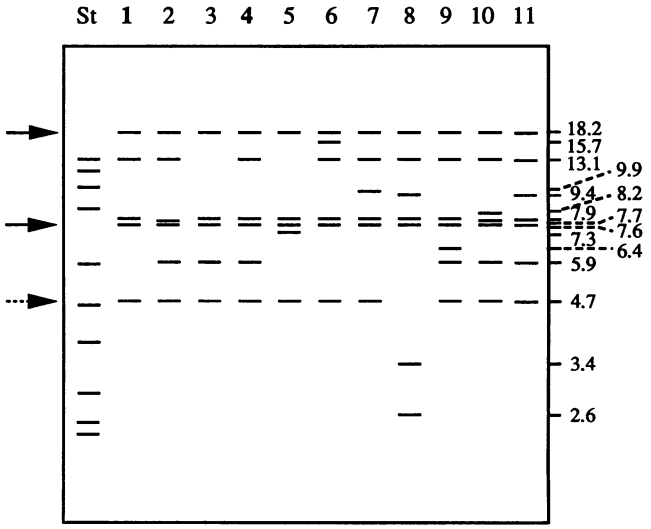

FIG. 2. Schematic representation of the 11 hybridization patterns obtained after cleavage of the genomic DNAs with *Eco*RI. St, molecular mass standards as described in the legend to Fig. 1. Boldface lane numbers indicate the two most frequent patterns. Numbers on the right indicate the molecular sizes (in kilobases) of the bands. Solid arrows on the left point to totally conserved bands, and the dotted arrow points to a band found in 69 of the 70 strains studied.

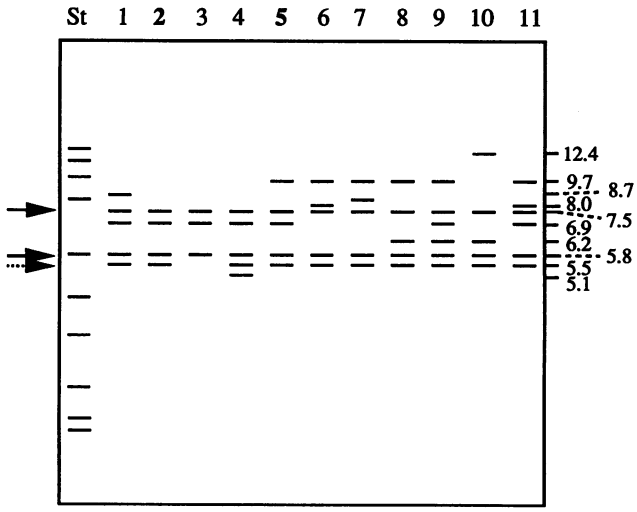

FIG. 3. Schematic representation of the 11 hybridization patterns obtained after cleavage of the genomic DNAs with *Eco*RV. For details, see the legend to Fig. 2.

When the *Eco*RI and *Eco*RV profiles were combined, 16 different ribotypes (A to P) were obtained (Tables 1 and 2). Each of nine ribotypes was found in only one strain. As expected, ribotypes B (RI.1 plus RV.2) and O (RI.4 plus RV.5) were found in 65.7% of the strains. However, the correlation between the results obtained with the two enzymes was not perfect, since a few strains which belonged to RI.1 or RI.4 did not belong to RV.2 or RV.5, respectively, and vice versa (Table 2).

Relationships between ribotypes and biovars of *Y. pestis*. A good correlation between the ribotypes and the biovars of the strains studied was established. The ribotypes common to two biovars were scarce: ribotypes F and G were found in both biovars Orientalis and Antiqua, and ribotype O was common to strains belonging to either biovar Medievalis or Antiqua. All other ribotypes were found exclusively in a single biovar: A to E in Orientalis, H to N in Antiqua, and P in Medievalis (Table 2).

TABLE 2. Breakdown of ribotypes among the 70 *Y. pestis* strains studied

*Eco*RI pattern	*Eco*RV pattern	Ribotype	No. of strains	Biovar
1	1	A	1	Orientalis
1	2	B	26	Orientalis
7	2	C	1	Orientalis
8	3	D	1	Orientalis
6	2	E	3	Orientalis
2	5	F	1	Orientalis
			2	Antiqua
1	4	G	2	Orientalis
			1	Antiqua
3	7	H	2	Antiqua
4	9	I	2	Antiqua
5	10	J	1	Antiqua
3	8	K	1	Antiqua
3	6	L	1	Antiqua
10	5	M	3	Antiqua
11	5	N	1	Antiqua
4	5	O	3	Antiqua
			17	Medievalis
9	11	P	1	Medievalis

Biovars Orientalis and Antiqua were heterogeneous; they were composed of 7 and 10 different ribotypes, respectively. In contrast, biovar Medievalis was much more homogeneous and contained almost exclusively ribotype O (17 of 18 strains).

Relationships between ribotypes and geographic origins of strains. Among the 26 strains of *Y. pestis* isolated in Africa, 12 different ribotypes were found, indicating a great heterogeneity of the isolates on this continent. In North Africa and Madagascar, almost all the strains isolated were of ribotype B. In contrast, this ribotype was not found in Central Africa (Kenya and Zaire [Belgian Congo]), but the ribotypes were heterogeneous (10 ribotypes for 17 strains) (Table 1).

The *Y. pestis* strains isolated in Asia were less heterogeneous than those from Africa; five ribotypes were found among 28 strains (Table 1). Furthermore, a certain local homogeneity was observed, i.e., ribotype G in Nhatrang, E in Saigon, B in Java, and O in Kurdistan.

Among strains from North and South America, only ribotype B was found (Table 1). However, these strains were isolated in only two countries, the United States and Brazil.

The only isolate from Oceania (Hawaii) exhibited ribotype B.

In the European *Y. pestis* strains (Hambourg), the heterogeneity of the ribotypes was high; there were four ribotypes for 5 strains (Table 1).

Analysis of the geographical distributions of the two most frequently observed ribotypes revealed that strains of ribotype B were isolated on all five continents, while those of ribotype O were isolated only in Central Africa and Central Asia.

Evaluation of the stabilities of the PFGE patterns (pulsotypes) within a given strain. We wanted to compare the efficiency of the pulsotype versus that of the ribotype in discriminating strains of *Y. pestis*. However, it was known that a large chromosomal deletion of approximately 100 kb occurs spontaneously in the chromosome of *Y. pestis* (8, 15). Furthermore, comparing the *Spe*I pulsotype of three isogenic colonies of strain Saigon 55-1239 which differed in their abilities to store hemin and to synthesize iron-regulated proteins, we observed that each colony exhibited a distinct profile with numerous varying restriction fragments (12). Therefore, prior to PFGE analysis of several strains of *Y. pestis*, we decided to evaluate the extent of variability of the pulsotype within one strain. For this purpose, eight colonies from the stock culture of strain Saigon 55-1239 were randomly picked, and their DNAs were subjected to PFGE. Five different *Spe*I restriction patterns were observed: one for colonies 1 and 3, one for colonies 5, 6, and 8, and a unique pattern for each of the three remaining colonies (Fig. 4). Similar differences were also observed in lower-molecular-mass fragments (data not shown), indicating a high heterogeneity of the pulsotypes within a given strain.

To ensure that these results were not limited to one strain, the same experiment was repeated with four colonies of strain Kenya 169. Three different *Spe*I pulsotypes were obtained (data not shown). Digestion of DNAs isolated from these colonies with other restriction enzymes, such as *Xba*I or *Not*I, gave similar results (data not shown).

In vitro stabilities of the ribotypes. The results obtained by PFGE prompted us to investigate whether such variability could also be observed in the ribotypes of *Y. pestis*. For this purpose, the *Eco*RI and *Eco*RV patterns of the eight colonies from strain Saigon 55-1239 were examined. The eight colonies had the same RI.6 and RV.2 patterns, which were identical to those of the parent strain (Fig. 5).

This experiment was repeated with five isolated colonies of

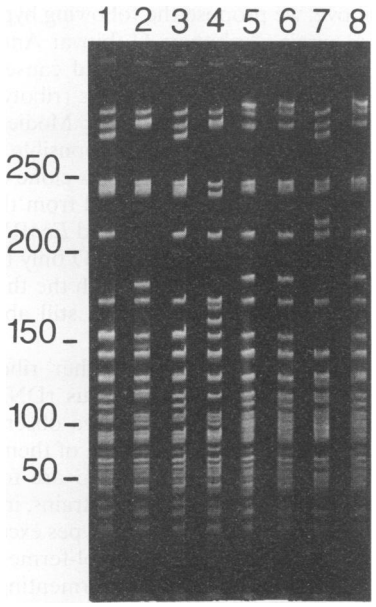

FIG. 4. *Spe*I restriction profiles of eight different colonies (lanes 1 to 8) from strain Saigon 55-1239 obtained with the zero integrated field electrophoresis method and with the ROM card allowing separation of fragments of 150 to 200 kb. Size markers (in kilobases; values have been rounded up) are indicated on the left.

strains Madagascar 62, Kenya 129, and Hambourg 13. Within each strain, the five colonies displayed the same *Eco*RI pattern, which was identical to that of the parental strain (data not shown).

In vivo stabilities of the ribotypes. Since numerous strains of *Y. pestis* had unique ribotypes, we wanted to evaluate the potential for evolution of the ribotypes in bacteria subjected to a selective pressure in vivo. Therefore, the virulent strain Madagascar 6/69 was injected into mice, recovered from the spleens, and reinjected in the same manner four additional times. The ribotypes of two isolated colonies were examined after each passage. Both the *Eco*RI (Fig. 6) and the *Eco*RV (data not shown) profiles of the two sets of colonies remained identical to those of the parental strain after each successive passage. These results indicate that the selective pressure exerted in vivo on the bacteria does not modify, at least not rapidly, the rRNA gene restriction sites.

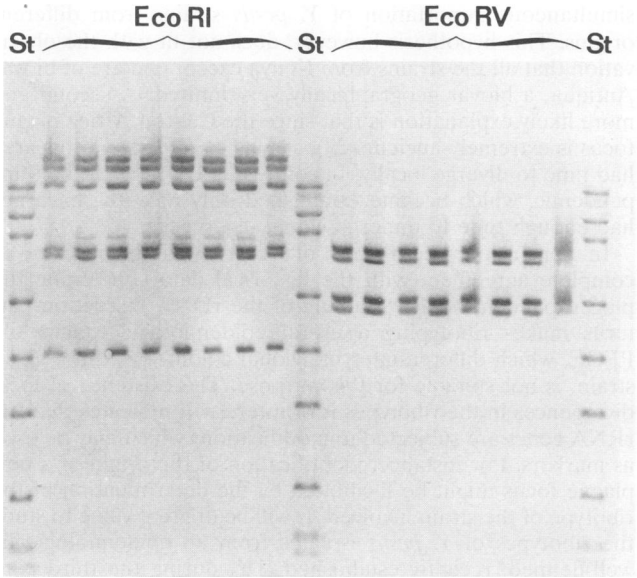

FIG. 5. Southern hybridization with the AAF-labeled 16S-23S rRNA probe of the *Eco*RI- or *Eco*RV-digested genomic DNAs of eight distinct isolated colonies from strain Saigon 55-1239. St, molecular mass standards as described in the legend to Fig. 1.

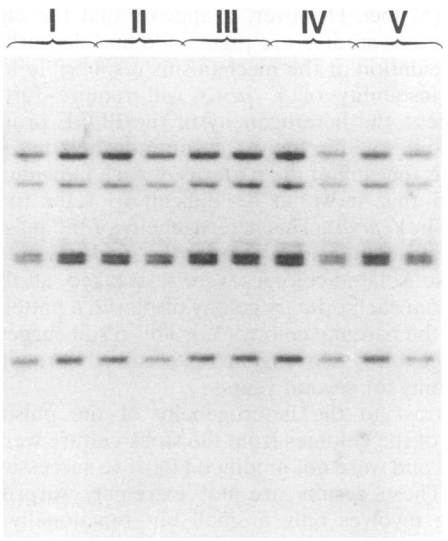

FIG. 6. Southern hybridization with the AAF-labeled rRNA probe of the *Eco*RI-cleaved genomic DNA of isolated colonies of strain Madagascar 6/69 recovered from the spleens of infected mice. Five in vivo passages (lanes I to V) were performed, and two colonies were examined each time.

DISCUSSION

The hybridization patterns with the *E. coli* 16S-23S rRNA probe of *Eco*RI- or *Eco*RV-digested *Y. pestis* DNA revealed the existence of 16 distinct ribotypes. This indicates that the species is not as homogeneous as was believed and that determination of the rRNA gene restriction pattern may be helpful in subdividing *Y. pestis* isolates.

Another method for analyzing the genomic heterogeneity of a bacterial species is PFGE. Comparing different strains of *Y. pestis* with this technique, Lucier and Brubaker (15) noted that each isolate exhibited a unique pattern of fragments but that the profiles of glycerol-negative bacteria were more similar to each other than to those of the glycerol-positive strains. Consistent with these findings, our results show a clear distinction in the ribotypes of glycerol-fermenting and -nonfermenting strains. However, while performing PFGE, we discovered that the genome of *Y. pestis* is subject to numerous and spontaneous genomic rearrangements which occur at a high frequency. Since *Y. pestis* harbors three well-known plasmids of ca. 9.5, 70, and 90 kb, it is possible that some of the variable fragments smaller than 100 kb correspond to plasmid bands. However, the heterogeneity observed among the fragments larger than 100 kb (Fig. 4) cannot be attributed to a variation in the plasmid content of the strains. One possible explanation may be that some of the strains underwent the high-frequency large chromosomal deletion involving the pigmentation (8, 15)

and *irp2* (5) loci. However, it appears that the eight Saigon 55-1239 strains studied are pigmented and do harbor the *irp2* gene. Elucidation of the mechanisms responsible for the high genomic instability of *Y. pestis* will require further study. Nevertheless, the heterogeneity of the PFGE profiles of different colonies within a given strain indicates that the pattern observed is the sum of the patterns of each individual bacterial clone and that it would be difficult to refer to a specific pulsotype in *Y. pestis*. This heterogeneity of the pulsotypes was observed in isolated colonies from the stock culture. However, when these isolated colonies were restreaked, all the colonies derived from each primary colony displayed a pattern identical to that of the parental colony (12). This result suggests that the observed genomic rearrangements occurred during the storage of the strains for several years.

In contrast to the heterogeneity of the pulsotypes, the ribotypes of the colonies from the stock culture were perfectly conserved and were not modified after five successive passages in vivo. These results are not extremely surprising, since ribotyping involves only a small but functionally important region of the chromosome, while PFGE is a representation of the whole genome. The fact that different pulsotypes are found within a given strain of *Y. pestis* renders the use of this technique for epidemiologic purposes questionable. In contrast, the stabilities of the ribotypes make the rDNA restriction pattern a much more adequate technique for these types of studies.

Y. pestis and *Y. pseudotuberculosis* constitute a single genomic species (3). However, the proposal to unite *Y. pestis* and *Y. pseudotuberculosis* in a single species has not been adopted because the clinical relevance of differentiating the two species prevailed over the taxonomic data. The *Eco*RI rRNA gene restriction patterns of several strains of *Y. pseudotuberculosis* have recently been determined (18). Comparison of these patterns with those of *Y. pestis* obtained in the present study indicates that they differ greatly. This finding reinforces the difference between *Y. pestis* and *Y. pseudotuberculosis* and suggests that ribotyping may help in differentiating the two organisms in some instances.

Analysis of the rRNA gene restriction patterns shows that two ribotypes predominate: of the 70 strains studied, 46 (65.7%) belong to either ribotype B (37.1%) or O (28.6%). It is noteworthy that the geographical distributions of these two ribotypes differ: B is found over the five continents, while O is restricted to Central Africa and Central Asia. Strikingly, the locations of strains of ribotype O correspond to the remaining plague foci of the first and second pandemics, while the distribution of ribotype B reflects the geographical spread of *Y. pestis* during the third pandemic (7, 19). It is therefore tempting to assume that a strain of ribotype B was responsible for the third pandemic.

By comparison of the two main ribotypes with the biovars of the isolates, it appeared that all the strains of ribotype B were of biovar Orientalis, while those of ribotype O were of either biovar Medievalis or biovar Antiqua. If nitrate reduction is not taken into account, there is a strict correlation between the ability to ferment glycerol and the ribotypes; none of the strains of ribotype B are able to ferment glycerol, while all those of ribotype O can ferment this sugar. Wu et al. (22) stated that plague has been present since time immemorial in the Central Asiatic plateau, which they considered the original home of the infection. Devignat (7) hypothesized that the biovars Antiqua and Medievalis might both have originated from this primary focus. The results of ribotyping are in complete agreement with this hypothesis.

On the bases of our present results and the work of the authors cited above, we propose the following hypothesis. One main clone of *Y. pestis* of ribotype O (biovar Antiqua) spread from Central Asia to Central Africa and caused Justinian's plague. Later, a variant of the same clone (ribotype O) which lost the ability to reduce nitrate (biovar Medievalis) spread from Central Asia to Crimea and was responsible for the Black Death. It seems probable that the *Y. pestis* clone of ribotype B which caused the third pandemic derived from the first clone for at least two reasons: (i) the *Eco*RI and *Eco*RV patterns of ribotype B differ from those of ribotype O only by the loss of one restriction fragment, and (ii) although the third clone has lost the ability to ferment glycerol, it is still able to reduce nitrate (biovar Orientalis).

The 24 strains which belong to neither ribotype B nor ribotype O have extremely heterogeneous rDNA restriction patterns. Indeed, 14 distinct ribotypes are observed, with no more than three strains belonging to each of them. In contrast to ribotypes B and D, ribotypes F and G are found in both glycerol-fermenting and -nonfermenting strains, indicating that the dichotomy is not perfect. All the ribotypes except these two are found exclusively either in glycerol-fermenting strains (ribotypes H to P) or in glycerol-nonfermenting strains (ribotypes A to E).

The origin of these low-frequency ribotypes is unclear. They might correspond to clonal divergences of local strains, as suggested by the three strains from Saigon, which are of ribotype E, a ribotype found nowhere else. Similarly, ribotype D is present only in Senegal, H, I, K, L, and M are present only in Kenya, N and J are present only in Zaire, and P is present only in Kurdistan. The existence of strains of ribotype G in Kenya and Nhatrang may indicate a common origin; however, the fact that the strains from these two countries do not have the same biovar may also indicate that a similar mutation occurred independently in the rDNAs of these two groups of bacteria.

One explanation for the great heterogeneity of ribotypes in the strains isolated in Kenya (eight ribotypes for 15 strains) might be that this country was subjected to large inland and maritime flows of merchandise and people and thereby to simultaneous importation of *Y. pestis* strains from different origins. This hypothesis however, does not fit with the observation that all the strains from Kenya except one are of biovar Antiqua, a biovar geographically very limited. A second and more likely explanation is that since the Central Africa plague focus is extremely ancient (22), strains of *Y. pestis* in this area had time to diverge locally. In contrast, the strains of the third pandemic, which became established only recently, have not had enough time to undergo clonal divergence.

In conclusion, the results of *Y. pestis* ribotyping are in complete agreement with the historical data concerning the plague pandemics. The stability of the rDNA restriction patterns makes ribotyping a useful epidemiologic tool, while PFGE, which differentiates individual colonies within a given strain, is not suitable for this purpose. The existence of local divergences in the ribotypes is of interest. It indicates that the rRNA genes are subjected to modifications which may be used as markers. For instance, identification of the origin of a new plague focus might be facilitated by the determination of the ribotype of the strain involved. It will be of great value to study the ribotypes of *Y. pestis* isolates from an epidemiologically well-defined, recently established (i.e., during the third pandemic) plague focus and to determine whether since the introduction of the strains local conditions exerted some selective pressure on them, resulting in the modification of their rRNA gene restriction patterns.

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REFERENCES

1. **Andersen, J. K., and N. A. Saunders.** 1990. Epidemiological typing of *Yersinia enterocolitica* by analysis of restriction fragment length polymorphisms with a cloned ribosomal RNA gene. J. Med. Microbiol. **32:**179–187.
2. **Baltazard, M.** 1971. Evolution de la recherche sur l'épidémiologie de la peste. Med. Mal. Infect. **1:**203–218.
3. **Bercovier, H., H. H. Mollaret, J. M. Alonso, J. Brault, G. R. Fanning, A. G. Steigerwalt, and D. J. Brenner.** 1980. Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridization and its relationship to *Y. pseudotuberculosis*. Curr. Microbiol. **4:**225–229.
4. **Blumberg, H. M., J. A. Kiehlbauch, and I. K. Wachsmuth.** 1991. Molecular epidemiology of *Yersinia enterocolitica* O:3 infections: use of chromosomal DNA restriction fragment length polymorphisms of rRNA genes. J. Clin. Microbiol. **29:**2368–2374.
5. **Carniel, E., A. Guiyoule, O. Mercereau-Puijalon, and H. H. Mollaret.** 1991. Chromosomal marker for the high pathogenicity phenotype in *Yersinia*. Contrib. Microbiol. Immunol. **12:**192–197.
6. **Carniel, E., O. Mercereau-Puijalon, and S. Bonnefoy.** 1989. The gene coding for the 190,000-dalton iron-regulated protein of *Yersinia* species is present only in the highly pathogenic strains. Infect. Immun. **57:**1211–1217.
7. **Devignat, R.** 1951. Variétés de l'espèce *Pasteurella pestis*. Nouvelle hyphothèse. Bull OMS **4:**247–263.
8. **Fetherston, J. D., P. Schuetze, and R. D. Perry.** 1992. Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol. Microbiol. **6:**2693–2704.
9. **Grimont, F., D. Chevrier, P. A. D. Grimont, M. Lefevre, and J. Guesdon.** 1989. Acetylaminofluorene-labelled ribosomal RNA for use in molecular epidemiology and taxonomy. Res. Microbiol. **140:**447–454.
10. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. (Paris) **137B:**165–175.
11. **Iteman, I., C. Baril, I. Saint Girons, and E. Carniel.** 1991. Pulse field electrophoresis of the chromosome of the pathogenic yersiniae. Contrib. Microbiol. Immunol. **12:**198–202.
12. **Iteman, I., A. Guiyoule, A. M. P. De Almeida, I. Guilvout, G. Baranton, and E. Carniel.** 1993. Relationship between loss of pigmentation and deletion of the chromosomal iron-regulated *irp2* gene in *Yersinia pestis*: evidence for separate but related events. Infect. Immun. **61:**2717–2722.
13. **Karimi, Y.** 1963. Conservation naturelle de la peste dans le sol. Bull. Soc. Pathol. Exotique **6:**1183–1186.
14. **Koblavi, S., F. Grimont, and P. A. D. Grimont.** 1990. Clonal diversity of *Vibrio cholerae* O1 evidenced by rRNA gene restriction patterns. Res. Microbiol. **141:**645–657.
15. **Lucier, T., and R. R. Brubaker.** 1992. Determination of genome size, macrorestriction pattern polymorphism, and nonpigmentation-specific deletion in *Yersinia pestis* by pulse-field gel electrophoresis. J. Bacteriol. **174:**2078–2086.
16. **Mollaret, H. H.** 1963. Conservation expérimentale de la peste dans le sol. Bull. Soc. Pathol. Exotique **6:**1169–1183.
17. **Najdenski, H., I. Iteman, and E. Carniel.** Unpublished data.
18. **Picard-Pasquier, N., B. Picard, S. Heeralal, R. Krishnamoorthy, and P. Goullet.** 1990. Correlation between ribosomal DNA polymorphism and electrophoretic enzyme polymorphism in *Yersinia*. J. Gen. Microbiol. **136:**1655–1666.
19. **Pollitzer, R.** 1954. Plague. W.H.O. Monogr. Ser. no. 22.
20. **Schaffer, H. E., and R. R. Sederoff.** 1981. Improved estimation of DNA fragment length from agarose gels. Anal. Biochem. **115:**113–122.
21. **World Health Organization.** 1993. Human plague in 1991. Bull. W.H.O. **4:**21–23.
22. **Wu, L.-T., J. W. H. Chun, R. Pollitzer, and C. Y. Wu.** 1936. Plague: a manual for medical and public health workers. Shangai, China.